Association of protein intake with bone mineral density and bone mineral content among elderly women: the OSTPRE Fracture Prevention Study.

Masoud Isanejad[1,3], Joonas Sirola[2,3], Jaakko Mursu [1], Heikki Kröger[2,3], Toni Rikkonen[3], Marjo Tuppurainen[4], Arja T Erkkilä [1].

[1]Institute of Public Health and Clinical Nutrition, University of Eastern Finland, P.O. Box 1627 Kuopio, Finland.

[2] Department of Orthopaedics and Traumatology, Kuopio University Hospital, Kuopio Finland

[3] Kuopio Musculoskeletal Research Unit, University of Eastern Finland, Kuopio, Finland

[4] Department of Obstetrics and Gynaegology, Kuopio University Hospital, Kuopio, Finland.

Address: Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Yliopistonranta 1C, PO Box 1627, FI70211 Kuopio, Finland.

Masoud Isanejad (corresponding author): *Address*: Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Yliopistonranta 1C, PO Box 1627, FI70211 Kuopio, Finland. *Phone number*: +358449·744684· *Email address*: masoud.isanejad@uef.fi.

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Abstract

It has been hypothesized that high protein intakes is associated with lower bone mineral content (BMC). Previous studies yield conflicting results and thus far no studies has undertaken the interaction of body mass index (BMI) and physical activity with protein intakes in relation to BMC and bone mineral density (BMD). **Objective**: To evaluate the associations of dietary total protein (TP), animal protein (AP) and plant protein (PP) intakes with BMC and BMD and their changes. We tested also the interactions of protein intake with, obesity (BMI ≤30 vs. >30 kg/m^2) and physical activity level (passive vs. active). **Design/ Setting**: Prospective cohort study (Osteoporosis Risk-Factor and Fracture-Prevention Study). **Participants/measures**: At the baseline, 554 women aged 65-72 years filled out a 3-day food record and a questionnaire covering data on lifestyle, physical activity, diseases, and medications. Intervention group received calcium 1000 mg/d and cholecalciferol 800 IU for 3 years. Control group received neither supplementation nor placebo. Bone density was measured at baseline and year 3, using dual energy x-ray absorptiometry. Multivariable regression analyses conducted to examine the associations between protein intake and BMD and BMC. **Results**: In cross-sectional analyses energy-adjusted TP (P≤0·029) and AP (P≤0·045) but not PP (g/d) were negatively associated with femoral neck (FN) BMD and BMC; women with TP≥1·2 g/kg/body weight (BW) (P_{trend}≤0·009) had lower FN, lumbar spine (LS) and total BMD and BMC. In follow-up analysis, TP (g/kg/BW) was inversely associated with LS BMD and LS BMC. The detrimental associations were stronger in women with BMI<30 kg/m^2. In active women, TP (g/kg/BW) was positively associated with LS BMD and FN BMC changes. **Conclusions**: This study suggests detrimental associations between protein intake and bone health. However, these negative associations were counteracted by BMI>30 kg/m^2 and physical activity.

Keywords: Dietary protein intake. Source of protein intake. Bone mineral density. Physical activity. Body mass index

Introduction

Osteoporosis is major public health problem, particularly in women (1). Bone mineral density (BMD) and bone mineral content (BMC) measured by dual energy x-ray absorptiometry (DXA), have been considered as important determinants of osteoporotic fractures (2). It is crucial to identify risk factors associated with low BMD due to its importance to fracture, functional quality of ageing as well as significant health costs (3). The role of dietary protein in bone health is unclear and also might be dependent on the presence of other factors (3-6). In meta-analysis by Darling et al. (6) for cross-sectional studies of protein intakes and BMD no association or a small positive association have been suggested. The source of protein consumed may be differentially associated with bone health in adults (7). It has been suggested that consumption of animal protein sources (AP) containing high acidifying amino acids might increase the risk of bone loss (8), while plant protein (PP) based diets contain isoflavones that may have protective effects on bone health (9). Further studies examining the sources of protein and their potential differentiating associations with bone health are warranted. Further understanding of the mechanisms behind how protein modifies bone metabolism, will provide future therapeutic targets in forestalling bone loss with aging (10, 11).

Protein might increase the protein-sensitive anabolic mediator of calcium such as insulin like growth factor (IGF-1) and increase intestinal calcium absorption (12, 13), whereas short term intervention study using purified protein supplements have shown that 1 mg calcium is on average lost in the urine for every 1 g increase in protein intake (14). However, whether bone is the source of this calcium loss has not been shown. Furthermore, body weight (BW) is an important determinant of BMD, individuals with higher BW have higher BMD and reduced fracture risk (15). Between-individuals variation in BW accounts for about 30% of variation in BMD, making it one of the strong determinants of BMD (16). Besides, it is evident from previous studies that physical activity has strong beneficial effect on bone health (17). It was shown also that physical activity and protein-containing supplement have positive effect on femoral neck (FN) BMD (18). However, whether greater physical activity combined with dietary protein are associated with increased BMD has not been investigated in cohort studies (19).

In this study, we evaluated the associations of total protein (TP), and protein intake by food source (AP and PP intakes) with BMD and BMC at lumbar spine (LS), FN and total body among elderly

80 women at the baseline and over 3 year of follow-up. We further tested the interaction of TP (g/kg
81 BW) with BMI and physical activity in relation to BMD and BMC.

Materials and methods

Study design and participants

Data of the present study were collected from the Osteoporosis Risk Factor and Fracture Prevention Study (OSTPRE-FPS), which was a 3-year intervention to investigate the effect of calcium and vitamin D supplementation on incidence of falls and fractures among elderly women. Inclusion criteria were being older than 65 years of age by the end of November 2002, residing in Kuopio region and no previous participation in OSTPRE bone densitometry sample. The intervention (supplementation) group (n=287) received daily cholecalciferol 800 IU (20 μg) and calcium 1000 mg for 3 years while the control group (n=306) received neither supplementation nor placebo (20). In total 750 women were randomly taken into this subsample for participating in detailed examinations including measurement of bone density and body composition and food records. Out of those, 554 returned valid food record and had valid body composition measurements for both at the baseline and after 3 year (21). All clinical measurements were performed in Kuopio Musculoskeletal research unit of the Clinical research center of the University of Kuopio, Kuopio, Finland. All participants provided written permission for participation. The study was approved in October 2001 by the ethical committee of Kuopio University Hospital. The study was registered in Clinical trials.gov by the identification NCT00592917.

Bone density measurements

BMC (g) was measured at the baseline and year 3, using DXA (Lunar Prodigy, Wisconsin, USA) for LS (L2-L4), FN and total body by trained nurses. BMD (g/cm^2) was calculated as BMC (g)/bone area (cm^2). DXA is a standard and the most widely used technique to determine BMD since the late 1980s (22). Technical quality of measurements was double checked and those with any measurement errors were excluded from the statistical analysis. The long-term reproducibility (CV) of the DXA instrument for BMD during the study period, as determined by regular phantom measurements, was 0·4% (20). Absolute changes in BMD and BMC were further calculated with the use of baseline and year 3 values. Height and weight of participants were measured in light indoor clothing without shoes, and body mass index (BMI) was calculated (kg/m^2).

Dietary intakes

Dietary intake was collected by using 3-day food record at the baseline. A questionnaire and instructions were sent to participants beforehand, and they were returned on the visiting day. Participants were advised to fill the questionnaire for 3 consecutive days, including 2 days during the week and one day in the weekend (Saturday or Sunday). In case of uncertainties in the food record, a nutritionist called the participant for additional information (23). To assess the underreporting the ratio of energy intake to estimated basal metabolic rate was calculated based on BW according to equations given by Department of Health in the UK (24). The ratio of energy intake to basal metabolic rate cutoff value for under-reporting was chosen to be 1·49, as derived from Goldberg et al.(25) and Black (26) and none of the participants was excluded from the analyses (27). Collected data provided calculations of AP (including egg, dairy, poultry and meat) and PP sources (including cereals, grains, vegetables and fruits) of protein in addition to TP intake. Nutritional intake from food was calculated using Nutrica program (version 2·5, Finnish social insurance institute, Turku, Finland).

Questionnaire

All lifestyle related information was gathered by the self-administered questionnaire. The questionnaire included questions on age, hormone therapy use (never used, used), time since menopause (years), smoking status (present status), self-reported calcium and vitamin D supplementation (yes, no) and alcohol consumption (portions/ week). Total exercise time/week was based on self-reported amounts and types of exercise/week. Participants were questioned also for their mobility status and categorized as no restriction, restricted and no mobility at the baseline. Diseases possibly affecting BMD included hyperthyroidism, disease of parathyroid gland, chronic liver disease, chronic intestinal disease, celiac disease, ventricle operation, chronic nephropathy arthritis, osteoporosis, and lactose intolerance. Medications that may influence BMD included loop-diuretics, insulin, antiepileptics, glucocorticoids and cancer chemotherapy (20).

Statistical analysis

All statistical analysis were executed using SPSS software version 21 for Windows (IBM Corp., Armonk, NY). Result was significant if a P value was $< 0{\cdot}05$. The protein intakes (TP, AP and PP) were adjusted for energy intake utilizing the residual method (28). An advantage of this

method is that it provides a measure of protein intake which is independent of total energy intake. Protein intake g/kg BW was calculated using crude protein intake divided per BW. Further, the selection of TP (g/kg BW) cut-offs were based on three different nutrition recommendations, RDA (29) (≤ 0·8 g/kg BW), PROT-AGE Study Group recommendation (30) (0·81-1·19· g/kg/BW), and Nordic Nutrition recommendation (≥ 1·2 g/kg BW) (31).

One way ANOVA was used to test differences in means of baseline characteristics of participants among quartiles of energy-adjusted protein intake. Each of the BMD and BMC measures at the baseline and changes in them over 3 year of follow-up were set as dependent variable in multiple linear regression or logistic regression models. Tests for a linear trend across categories of protein intake (g/kg BW) were conducted by using the median value in each category of protein intake as a continuous variable in the linear and logistic regression models.

Model 1 was adjusted for age, energy intake, height, weight, and study group (intervention calcium and vitamin D). Model 2 was further adjusted for variables in model 1 plus dietary calcium and vitamin D intake, self-reported vitamin D and calcium supplementation, smoking status, physical activity level, hormone therapy use, time since menopause (years), diseases and use of medications which affect BMD. BMD and BMC variables at the baseline were entered in longitudinal models as an independent variable to account for differential subsequent changes of BMD and BMC depending on initial measures. AP and PP intakes were included in the same regression model to adjust for each other. To manage the strong collinearity of the protein intake as expressed per BW (dependent variable) and BW as covariate, in analysis using TP (g/kg BW), BW was dropped from the adjusted covariates (32, 33).

Subgroup analysis

We tested the interaction of TP (g/kg BW) with obesity and physical activity level. Obesity was defined using WHO criteria where women with BMI >30 g/kg m^2 were categorized as obese (34). The physical activity level was compiled from frequency of exercise times per week and mobility status. Women were classified as passive if they had restricted or no mobility and exercise ≤ 2 times/week and those with no mobility restriction and exercise > 2 times/week were classed as active. Interactions between TP intake g/kg BW with obesity status (BMI ≤ 30 and > 30 kg/m^2) and physical activity level (passive/active) were tested by introducing an interaction term in model 2. In this data total intake of calcium at the baseline did not predict annual BMD changes (20).We

also checked for the interaction of dietary calcium intake, self-reported calcium supplement and total calcium intake (dietary + self-reported calcium supplement) with protein intake in relation to BMD and BMC, and associations were not significant.

Results

The mean age was 68·1 (SD 1·9) years, and mean energy intake was 6560 (SD 1556) kJ/d (Table 1). Total protein intake was 68·2 g/d which constituted 17% of total energy intake and corresponded to 0·96 g/kg BW. Women in the second and fourth quartiles of energy-adjusted TP intakes had significantly higher BW. Women in the first and third quartiles of TP intake reported more use of HT (46%) as compared to women in the second and fourth quartiles. Those in the third quartile had higher percentage of participation in calcium and vitamin D interventional supplementation and also had higher self-reported vitamin D supplementation.

Total energy intake (kJ/d), dietary calcium and total calcium intake (mg/d) were significantly higher in higher quartiles of protein intake and total fat intake (g/d) was highest in the fourth quartile. TP and AP intakes were significantly higher in higher quartiles of protein intake, while no significant association was observed for PP intake. Dietary carbohydrate (g/d) and phosphorus (mg/d) intakes were highest in the first quartile and dietary magnesium intake (mg/d) increased by higher protein intake. Mean BMD at the baseline was 1·096 g /cm² (T-score: -0·78), 0·869 g /cm² (T-score: -0·924) and 1·077 g /cm² (T-score: -0·603) for LS, FN and total body, respectively. In 3 years of follow up FN BMD decreased by -1·89%, while LS and total body BMD increased by +0·93% and +0·56%, respectively.

Cross-sectional BMD and BMC

At the baseline in model 2 energy adjusted TP ($\beta \geq -0{\cdot}19$ and $P \leq 0{\cdot}029$) and AP ($\beta \geq -0{\cdot}02$ and $P \leq 0{\cdot}029$) were negatively associated with FN BMD and FN BMC, while no such association was observed for PP intake (**Table 2**). Further, TP (g/kg BW) ($\beta \geq -0{\cdot}28$ and $P \leq 0{\cdot}009$) was in negative associations with FN, LS and total BMD and BMC. Similar results were observed using categories of protein intake (g/kg BW) where women with higher protein intake ≥ 1·2g/kg BW had the lowest LS, FN and total BMD and BMC at the baseline (data not shown).

Longitudinal changes in BMD and BMC

Results for the prospective analysis are presented in total population in **Table 3**. The interactions between energy-adjusted TP, AP and PP intakes (g/d) as well as TP (g/kg BW) and interventional vitamin D and calcium supplementation were not significant ($P \geq 0{\cdot}660$) so groups are kept together. In the prospective analysis in model 2, TP intake (g/kg BW) was negatively associated with changes of LS BMD and LS BMC ($\beta \geq -0{\cdot}30$ and $P \leq 0{\cdot}002$).

Protein and BMI interaction

The interaction between protein intake and BMI was significant only for association with FN and LS BMC ($P_{interaction} \leq 0{\cdot}007$). At the baseline, in women with BMI ≤ 30 kg/m^2, TP (g/kg BW) was negatively associated with LS and FN and total BMD ($\beta \geq -0{\cdot}25$ and $P \leq 0{\cdot}050$) as well as FN and total BMC ($\beta \geq -0{\cdot}31$ and $P \leq 0{\cdot}007$) (**Table 4**). In prospective analysis, among women with BMI ≤ 30 kg/m^2, TP intake (g/kg BW) was negatively associated with change of LS BMD ($\beta = -0{\cdot}31$ and $P = 0{\cdot}016$).

Protein and physical activity interaction

Association of TP (g/kg BW) at the baseline and over 3 year of follow-up was further explored according to physical activity level of the participants (**Table 5**). Interaction between TP and physical activity level was significant only in association with total BMC and BMD ($P_{interaction} \leq 0{\cdot}050$). At the baseline TP (g/kg BW) was negatively associated with FN BMD ($\beta \geq -0{\cdot}26$ and $P \leq 0{\cdot}041$) and FN BMC ($\beta \geq -0{\cdot}22$ and $P \leq 0{\cdot}036$) in both physically passive and active women. In prospective analysis, among passive women TP (g/kg BW) was negatively associated with LS BMD and LS BMC loss ($\beta \geq -0{\cdot}43$ and $P \leq 0{\cdot}003$), while among active women TP (g/kg BW) was in positive relationships with changes of LS BMD ($\beta = 0{\cdot}23$ and $P = 0{\cdot}047$) and FN BMC ($\beta = 0{\cdot}21$ and $P = 0{\cdot}049$) over 3 years of follow-up.

Discussion

In our data at the baseline energy-adjusted TP (g/d) and AP (g/d) but not PP (g/d) were negatively associated with FN BMD and BMC. Women with higher protein intake (g/kg BW) also had lower FN, LS and total BMD and BMC. In follow-up analysis TP (g/ kg BW) was associated with loss of LS BMD and LS BMC. To the best of our knowledge this is the first cohort study which focused on different modifiers in association of protein intake with BMD and BMC. We evaluated and

suggested that association of dietary protein intake with bone density may differ according to participants' lifestyle characteristics. TP (g/kg BW) negatively associated with BMD and BMC only in women with BMI $\leq 30 kg/m^2$, and it was in positive relationship with changes of LS BMD and FN BMC in active women. These findings were observed independent of relevant covariates and confounders.

Most of the previous cross-sectional observational studies reported positive association between protein intake and higher BMD (6, 7, 35) or did not detect detrimental associations (36, 37). Findings by Sahni et al.(35) showed that protein intake was positively associated with FN, trochanter and LS BMD in women, while no significant associations were seen in men at any bone site. In contrast, in study by Darling et al.(38) in 176 postmenopausal women (aged 58 years and older) protein intake was negatively associated with LS and FN BMD as well as FN BMC.

Protein intake from different dietary sources may influence bone health by different mechanisms, including increasing calcium absorption or regulating plasma IGF-1 that increases bone formation (38, 39). PP based diets contain isoflavones that may have protective effects on bone health, however, their protective effects were not observed when used as dietary supplementation (9). AP sources contain more sulphur-containing amino acids such as methionine and cystein as compared to PP sources that can release protons which may decrease the pH and therefore increase the bone dissolution and bone loss (38, 40, 41). Previous epidemiological studies regarding association of PP and AP intakes and BMD have reported inconsistent results (3, 4, 8, 42, 43). Among white women (aged 80 years or older), higher PP intake was associated with higher BMD, while there were no consistent significant associations for TP and PP intakes among white women or other sex and racial/ethnic groups (42). In this data AP but not PP was negatively associated with FN BMD and BMC. Further investigations are warranted to evaluate whether AP and PP intakes have different associations with bone health.

Different study designs and population, including the length of follow-up, predominant protein sources of the diet, calcium content, lifestyle factors as well as discrepancies in data reporting, can all lead to inconsistency of the results of previous studies regarding the relationship of protein intake with bone health (4, 44). Given that we observed negative associations for protein intakes and BMC and BMD, stratified analysis was conducted to evaluate whether BMI and physical activity level mediate these associations. In postmenopausal elderly women BW and BMI are

strongly associated with bone health through weight bearing (15, 45, 46). Several data indicated that women with high BMI (25·0-29·9 kg/m^2) are protected from osteoporosis (47). Recent findings by Yang et al. in 5287 men and women aged between 8-69 years showed that greater BMI was associated with increased LS and FN BMD (48). However, it has been suggested that BMI > 30 kg/m^2 may be harmful to bone health (46). In this study negative associations of protein intake and BMD and BMC were more pronounced in those with BMI ≤ 30 kg/m^2 as compared to their counterparts with BMI > 30 kg/m^2. Mean protein intake did not differ between women with BMI ≤ 30 and BMI >30 kg/m^2 (17·4 % and 17·8 % of energy, respectively). Findings by Rikkonen et al.(49) in this population also showed that women with osteoporosis (FN BMD T score ≤ 2·5 SD) had a lower BMI, lower lean mass, but not fat mass proportion as compared to their normal counterparts. However, for the interaction between protein intakes with obesity, muscle mass and bone health more investigations are required.

It is evident from previous studies that physical activity has strong beneficial effect on bone health (17). In a 6-month, RCT in 19 healthy early postmenopausal women allocated to either postexercise consumption of a protein-containing nutrient supplement (with additional calcium and vitamin D) or a placebo supplement (with minimal energy); results revealed that there was a positive effect of the protein-containing supplement on FN BMD (18). However, trials are limited by short durations and small sample sizes. Results of the present study demonstrated that at the baseline protein intake (g/kg BW) was inversely associated with FN BMD and BMC in both passive and active women. While, follow-up results showed that in passive women protein intake (g/kg BW) was negatively associated with changes of LS BMD and BMC while in active women protein intake (g/kg BW) was in positive relationships with changes of FN BMD and BMC. Therefore, this data suggests that the interaction of physical activity and dietary protein might have positive relationship with bone density in elderly women. To our knowledge this was the first cohort study in elderly women exploring the exercise combined with dietary protein association and bone health and further studies are warranted.

Current study contains also some limitations. The 3-day dietary records method has been described as a suitable instrument for assessing energy and protein intake in elderly people (50, 51) , which has been also used and applied to measure AP and PP intake (52) . However, a single 3 day dietary record at the baseline might not be appropriate method to capture long term protein intake. Albeit

we covered a wide selection for several known confounders that might influence BMD and BMC, other factors might have affected the observed results. Participants who took part in an osteoporosis study may have had a heightened awareness of their bone health. This may have led them to alter some of their modifiable osteoporosis risk factors between the baseline and follow-up visits. However, such an effect is unlikely to have influenced protein consumption; since protein is not commonly perceived to be an osteoporosis risk factor. We cannot exclude also the possible effect of body composition on BMD background (53). Likewise to other studies observed effects in longitudinal analyses were weaker than what would be predicted by cross-sectional assessments. Lastly, based on the observational nature of our study we cannot establish a causal association.

Observed results could be confounded by mechanical errors. Fat mass loss during weight loss can affect tissue thickness and bone area measurements; therefore, present study reported both BMD and BMC (54). The availability of each BMD and BMC measures at the baseline as well as over a 3 year period added significant strength to our study. The analyses were adjusted for total energy intake and protein was reported as energy-adjusted and expressed as per BW, therefore, results showed separated effect of protein intake on BMD and BMC independent of the intake of energy from other sources.

Conclusion

Findings of the present study suggest that protein intake g/d and g/kg BW were negatively associated with BMD and BMC. This study highlights the importance of higher BMI and physical activity in counteracting the adverse association of protein intake and bone health. However, due to several unestablished aspects of these interactions, further cohort and intervention studies are warranted.

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Table 1. Baseline characteristics of participants across quartiles of energy-adjusted total protein intake (g/d).

Characteristics	Q 1 (<54·73 g/d) n=138		Q 2 (54·73-66·0 g/d) n=139		Q 3 (66-80·3 g/d) n=139		Q 4 (>80·3 g/d) n=138		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*
Age (years)	68·1	1·9	67·9	1·8	67·6	1·7	67·8	1·9	0·078
Weight (kg)	71·2	12·2	73·7	11·9	71·5	11·3	73·4	12·7	0·014
Height (cm)	157·9	5·6	158·4	5·5	159·4	4·8	158·7	5·3	0·139
BMI (kg/m^2)	27·2	4·6	26·8	3·6	27·8	4·1	28·0	4·2	0·085
Current smoker (%)	7·5		4·4		4·3		2·9		0·194
Portions of alcohol/week (n)	3·0	0·7	2·9	0·6	3·0	0·6	4·4	0·7	0·081
Physical activity level (%) [b]									0·660
Passive	39·1		33·8		40·3		39·9		
Active	60·9		66·2		59·7		60·1		
Hormone therapy use (%)	46·0		41·3		46·0		41·3		0·008
Interventional calcium and vitamin D supplement (%)	14·5		26·8		30·2		21·2		0·010
Disease or medication affecting bone (%)	38·4		33·1		37·0		37·0		0·816
Bone measurements									
Baseline total BMD	1·06	0·93	1·07	0·92	1·07	0·86	1·08	0·99	0·988
Baseline FN BMD	0·85	0·11	0·87	0·11	0·85	0·11	0·84	0·11	0·383
Baseline lumbar BMD	1·08	0·17	1·09	0·19	1·06	0·14	1·08	0·19	0·797
Baseline total BMC	2·12	0·34	2·23	0·57	2·21	0·30	2·24	0·32	0·832
Baseline FN BMC	4·11	0·57	4·22	0·31	4·14	0·59	4·15	0·60	0·320
Baseline lumbar BMC	4·30	0·11	4·41	0·12	4·22	0·91	4·45	0·11	0·723
Dietary intakes									
Total energy (kJ/d)	5091	1108	6150	1071	6907	1037	8083	1238	0·036
Fat (g/d)	55·6	9·9	54·1	10·1	51·3	8·9	66·8	17·6	0·005
Carbohydrate (g/d)	204·0	51·5	190·5	45·5	187·6	48·0	193·3	47·8	0·028
Protein (g/d)	47·0	7·7	60·6	3·2	72·7	4·3	92·0	10·5	<0·001
Animal protein (g/d)	24·7	5·9	35·2	2·0	42·5	2·4	54·3	6·7	<0·001
Plant protein (g/d)	23·5	4·4	24·0	4·5	24·4	4·0	24·1	4·2	0·451
Protein g/ kg body weight	0·79	0·24	0·90	0·23	0·96	0·27	1·18	0·29	<0·001
Magnesium (mg/d)	311·4	74·5	323·8	66·6	339·9	67·6	371·4	69·0	<0·001
Phosphorus (mg/d)	357·9	48·7	296·1	43·2	329·8	44·0	315·3	42·57	<0·001
Dietary calcium intake (mg/d)	799·4	317·6	908·2	285·3	1077·8	308·9	1257·7	385·9	0·001
Total calcium (mg/d) [c]	879·6	318·1	981·1	344·3	1187·1	358·9	1341·4	392·0	0·001
SR Calcium supplement (%)	20·3		24·6		31·7		27·7		0·170
SR vitamin D supplement (%)	14·5		26·8		30·2		21·2		0·010

Abbreviations: BMD, bone mineral density. FN, femoral neck. SD, standard deviation. SR, self-reported.
[a] ANOVA or chi-square tests were used to evaluate the distribution. [b] Passive: those women with restricted or no mobility and exercise ≤ 2 times/week. Active: those women with no mobility restriction and exercise > 2 times/week were classed as active. [c] Total calcium consists of dietary calcium and SR calcium supplement.

Table 2. Cross-sectional association between protein intake and BMD (g/cm^2) and BMC (g).

	FN BMD			LS BMD			Total BMD			FN BMC			LS BMC			Total BMC		
	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*
Total protein (g/d)																		
Model 1 [a]	-0·09	0·01	0·094	-0·05	0·01	0·366	-0·01	0·01	0·794	-0·06	0·01	0·186	-0·01	004	0·875	-0·01	1·23	0·979
Model 2 [b]	-0·19	0·01	0·029	-0·08	0·01	0·307	-0·11	0·01	0·185	-0·19	0·01	0·018	-0·06	0·07	0·943	-0·05	2·07	0·480
Animal protein (g/d) [c]																		
Model 1	-0·09	0·01	0·093	-0·04	0·01	0·364	-0·01	0·01	0·790	-0·06	0·01	0·185	-0·01	0·04	0·867	-0·01	1·23	0·978
Model 2	-0·20	0·01	0·029	-0·09	0·01	0·307	-0·01	0·01	0·185	-0·02	0·01	0·018	-0·01	0·07	0·943	-0·05	2·07	0·480
Plant protein (g/d) [c]																		
Model 1	-0·07	0·01	0·194	-0·03	0·01	0·599	-0·02	0·01	0·668	-0·04	0·01	0·367	-0·02	0·11	0·700	-0·02	3·39	0·608
Model 2	-0·06	0·01	0·325	-0·01	0·01	0·821	-0·01	0·01	0·790	-0·05	0·01	0·411	-0·01	0·14	0·989	-0·03	4·02	0·487
Total protein (g/kg body weight) [d]																		
Model 1	-0·23	0·03	0·001	-0·23	0·04	0·002	-0·25	0·02	0·001	-0·23	0·03	0·001	-0·18	2·47	0·009	-0·26	72·9	<0·001
Model 2	-0·39	0·04	0·001	-0·36	0·06	0·001	-0·51	0·03	<0·001	-0·38	0·21	<0·001	-0·28	3·80	0·009	-0·47	10·61	<0·001

Abbreviations: BMD, bone mineral density. FN, femoral neck. LS, lumbar spine. TP, total protein. AP, animal protein. PP, plant protein. SE, standard error.

[a] Model 1 was adjusted for age, total energy intake, height (cm), weight (kg) and study group.

[b] Model 2 was adjusted for variables in model 1 plus dietary vitamin D, dietary calcium intake, self-reported vitamin D and calcium supplementation, smoking status(current, former and nonsmokers), physical activity level (passive and active), hormone therapy use (never used, used), time since menopause (years); diseases and use of medications which affect BMD.

[c] Models for animal protein were also adjusted for plant protein intake. Models for plant protein were also adjusted for animal protein intake.

[d] Body weight was excluded from adjusted variables in analysis using protein as expressed per body weight due to high collinearity. However, result remained significant even after controlling for body weight.

Table 3. Prospective association of protein intake and changes in BMD (g/cm^2) and BMC (g).

	FN BMD			LS BMD			Total BMD			FN BMC			LS BMC			Total BMC		
	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*	β	SE	*P*
TP (g/d)																		
Model 1 [a]	0·07	0·01	0·077	0·05	0·01	0·273	0·11	0·01	0·044	0·08	0·01	0·050	0·07	0·01	0·138	0·03	0·36	0·505
Model 2 [b]	0·08	0·01	0·239	-0·03	0·01	0·617	0·12	0·01	0·174	0·10	0·01	0·164	-0·06	0·02	0·420	-0·08	0·58	0·064
AP (g/d) [c]																		
Model 1	0·08	0·01	0·056	0·08	0·01	0·075[†]	0·11	0·01	0·035	0·09	0·01	0·038	0·07	0·01	0·110	0·04	0·35	0·442
Model 2	0·10	0·01	0·160	0·03	0·01	0·712	0·17	0·01	0·077	0·12	0·01	0·123	-0·04	0·02	0·569	-0·05	0·59	0·531
PP (g/d) [C]																		
Model 1	-0·07	0·01	0·095	-0·10	0·01	0·075	-0·09	0·01	0·070	-0·07	0·01	0·091	-0·05	0·03	0·247	-0·10	0·95	0·053
Model 2	-0·05	0·01	0·301	-0·11	0·01	0·066	-0·14	0·01	0·054	-0·04	0·01	0·409	-0·04	0·04	0·492	-0·08	1·10	0·208
TP (g/kg body weight) [d]																		
Model 1	0·02	0·01	0·692	-0·14	0·01	0·038	0·05	0·01	0·471	0·09	0·05	0·141	-0·09	0·70	0·168	-0·01	21·12	0·928
Model 2	-0·01	0·01	0·918	-0·31	0·01	0·001	0·04	0·01	0·507	0·16	0·07	0·083	-0·30	1·02	0·002	-0·16	30·04	0·159

Abbreviations: BMD, bone mineral density. FN, femoral neck. LS, lumbar spine. TP, total protein. AP, animal protein. PP, plant protein. SE, standard error.

[a] Model 1 was adjusted for age, total energy intake, height (cm), weight (kg), study group and baseline BMD and BMC values .

[b] Model 2 was adjusted for variables in model 1 plus dietary vitamin D, dietary calcium intake, self-reported vitamin D and calcium supplementation, smoking status (current, former and nonsmokers), physical activity level (passive and active), hormone therapy use (never used, used), time since menopause (years); diseases and use of medications which affect BMD.

[c] Models for animal protein were also adjusted for plant protein intake. Models for plant protein were also adjusted for animal protein intake.

[d] Body weight was excluded from adjusted variables in analysis using protein as expressed per body weight due to high collinearity. However, result remained significant even after controlling for body weight.

Table 4. Cross-sectional and prospective association of protein intake (g/kg body weight) and BMD (g/cm^2) and BMC (g) according to BMI category.

	BMI ≤ 30 kg/m^2 (n=401)			BMI > 30 kg/m^2 (n=151)		
	β	SE	P [a]	β	SE	P
Lumbar spine BMD (g/cm^2)						
Baseline	-0·25	0·08	0·050	0·31	0·27	0·472
Change	-0·31	0·02	0·016	-0·05	0·05	0·778
Femoral neck BMD (g/cm^2)						
Baseline	-0·34	0·05	0·006	-0·12	0·27	0·776
Change	0·03	0·01	0·802	-0·01	0·04	0·940
Total BMD (g/cm^2)						
Baseline	-0·38	0·04	0·002	0·28	0·17	0·518
Change	0·02	0·01	0·869	-0·19	0·05	0·694
Lumbar spine BMC (g)						
Baseline	-0·16	4·42	0·191	0·22	16·183	0·525
Change	-0·21	1·38	0·104	-0·19	2·88	0·314
Femoral neck BMC (g)						
Baseline	-0·31	0·24	0·007	-0·23	1·41	0·551
Change	0·12	0·08	0·299	0·09	0·30	0·601
Total BMC (g)						
Baseline	-0·41	120·99	<0·001	-0·06	686·71	0·877
Change	-0·21	32·24	0·100	0·39	207·94	0·425

Abbreviations: BMD· bone mineral density, BMD, bone mineral density. BMC, bone mineral content.

[a] Model was adjusted for age, total energy intake, height, study group, dietary vitamin D and calcium intakes, self-reported vitamin D and calcium supplementation, smoking status (current, former and nonsmokers), physical activity level (passive and active), hormone therapy use (never used, used), time since menopause (years); diseases and use of medications which affect BMD and baseline BMD and BMC values for longitudinal analysis.

Table 5. Cross-sectional and prospective association of protein intake (g/kg body weight) and BMD (g/cm^2) and BMC (g) according to physical activity level.

	Passive (n=211)			Active (n=341)		
	β	SE	P [a]	β	SE	P
Lumbar spine BMD (g/cm^2)						
Baseline	0·01	0·16	0·963	-0·20	0·10	0·268
Change	-0·43	0·02	0·003	0·23	0·02	0·047
Femoral neck BMD (g/cm^2)						
Baseline	-0·26	0·06	0·041	-0·30	0·04	0·006
Change	-0·16	0·02	0·264	0·13	0·01	0·467
Total BMD (g/cm^2)						
Baseline	-0·11	0·07	0·590	-0·26	0·05	0·134
Change	-0·07	0·01	0·678	0·024	0·01	0·882
Lumbar spine BMC (g)						
Baseline	0·07	9·61	0·732	-0·10	5·90	0·578
Change	-0·46	1·50	0·002	0·20	1·40	0·125
Femoral neck BMC (g)						
Baseline	-0·22	0·30	0·036	-0·31	0·21	0·004
Change	-0·02	0·14	0·840	0·21	0·08	0·049
Total BMC (g)						
Baseline	-0·05	2·47	0·788	-0·12	1·62	0·435
Change	-0·11	55·40	0·545	0·24	38·72	0·146

Abbreviations: BMD, bone mineral density. BMC, bone mineral content.

[a] Model was adjusted for age, total energy intake, height, weight, study group, dietary vitamin D and calcium intakes, self-reported vitamin D and calcium supplementation, smoking status (current, former and nonsmokers), hormone therapy use (never used, used), time since menopause (years); diseases and use of medications which affect BMD and baseline BMD and BMC values for longitudinal analysis.